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Calcium-dependent enhancement of transcription of p300 by human T-lymphotropic type 1 p12[I]

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Abstract

Human T-lymphotropic virus type 1 (HTLV-1) p12[I] localizes to the endoplasmic reticulum and Golgi causing sustained release of calcium, T cell activation, and enhanced expression of several calcium-regulated genes. In recent microarray studies, p300 mRNA was increased in T cells expressing p12[I]. The co-activator p300 is a key regulator of cellular and viral transcription; however, factors that influence its transcriptional regulation are less well studied. We hypothesized that the transcription of p300 is calcium dependent and that sustained low magnitude increases in intracellular calcium may enhance the transcription of p300. Herein, we report enhanced expression of p300 in T cells by p12[I] in a calcium-dependent, but calcineurin-independent manner. Sustained low magnitude calcium release induced by ionomycin in T cells was sufficient to increased mRNA and protein levels of p300 resulting in enhanced transcription from a p300-dependent promoter. Promoter analysis of the p300 gene was used to predict calcium-responsive transcription factor binding sites. Using mutant forms of p12[I], we demonstrate that ER localization of the viral protein is required to increase p300. In addition, p12[I] reversed the repression of HTLV-1 LTR-driven transcription by HTLV-1 p30[II], a p300-binding protein. HTLV-1 p12[I]-mediated enhancement of p300 expression represents a novel mechanism of regulation of cellular gene expression by viral proteins. By targeting a ubiquitous second messenger such as calcium, HTLV-1 p12[I] may regulate the expression of the cellular transcriptional co-activator p300 to modulate viral gene expression and promote lymphocyte survival.

Keywords: Calcium; p300; Retrovirus; HTLV-1; Accessory protein; Transcription

Introduction

p300 is an important regulator of the transcription apparatus in response to a variety of cell signaling pathways that determines cell proliferation, differentiation, and apoptosis. As a transcriptional co-activator, p300 interacts with various cellular and viral promoter elements by bridging together transcription factors or modifying, through histone acetyltransferase activity, the structure of nucleosomal histones (Chan and La Thangue, 2001). A large number of sequence-specific, DNA-binding factors form complexes with p300, including nuclear steroid receptors, c-Jun, Fos, p53, Sap1, Stat1 and Stat2, MyoD, Ets-1, NFκB, HIF1, GATA 1, cMyb, and Smad proteins (Blobel, 2002; Iyer et al., 2004). In addition, p300 interacts with TBP, TFIIB, TFIID, RNA helicase A, CREB, MAP kinase p90rsk, and RNA polymerase II (reviewed in Janknecht, 2002; Goodman and Smolik, 2000; Vo and Goodman, 2001). Several viral proteins also interact with p300, including HTLV-1 p30[II] and Tax, adenovirus E1A, HIV-1 Vpr and Tat, Kaposi's

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sarcoma-associated herpes virus (KSHV) viral interferon regulatory factor protein (vIRF), simian virus 40 large T antigen, HPV E6 and E7, small delta antigen of hepatitis delta virus, Epstein–Barr virus (EBV) nuclear antigen 3C (EBNA3C) and EBNA2, and herpes simplex virion protein-16 (VP16) (Ludlow and Skuse, 1995; Marzio and Giacca, 1999; Goodman and Smolik, 2000; Ali and Decaprio, 2001; Kashanchi and Brady, 2005; Zhang et al., 2001).

p300 mediates the activities of various transcription factors; however, its availability in the cell is limited (Petrij et al., 1995). A variety of cellular proteins are known to compete with each other for binding to p300 (Colgin and Nyborg, 1998). This environment of competition between transcription factors for co-activator binding provides an additional layer of tightly regulated gene expression. Competition between viral and cellular proteins for binding p300 has been reported for HIV, adenovirus, and SV40 (Hottiger et al., 1998; Yang et al., 1996). The activities of p300 are regulated through competitive protein–protein interactions or post-translational modifications such as phosphorylation and acetylation (Chan and La Thangue, 2001; Giordano and Avantaggiati, 1999). However, the transcriptional regulation of p300 remains to be elucidated.

A doubly spliced mRNA within the most distal 3′ end of the HTLV-1 genome encodes both positive regulators of viral gene expression, the Rex and Tax proteins from ORFs III and IV, respectively (reviewed in Younis and Green, 2005; Grassmann et al., 2005; Kashanchi and Brady, 2005). In HTLV-1, both the singly and doubly spliced ORF I mRNAs encode a protein of 12 kDa ($p12^I$) (Ciminale et al., 1992; Koralnik et al., 1993). A unique doubly spliced mRNA from ORF II encodes $p30^{II}$ (Ciminale et al., 1992), whereas a singly spliced mRNA within the same ORF II encodes $p13^{II}$ (Berneman et al., 1992; Koralnik et al., 1993). Nucleotide sequence alleles of $p12^I$ including those that would result in specific residue changes associated with protein stability (lysine versus arginine at position aa 88) or truncation mutants have been reported in a minority (<7%) of HTLV-1-infected subjects with HAM/TSP, ATL, and asymptomatic patients (Martins et al., 2002; Furukawa et al., 2004; Iniguez et al., 2006). The influence of these HTLV-1 variants in viral transmission or disease outcomes is unclear. We have reported that ablation of the acceptor splice site for the singly and doubly spliced ORF I mRNA is associated with a reduction of viral infectivity in vivo (Collins et al., 1998) that can be modeled when resting T cells are used as targets in vitro (Albrecht et al., 2000).

$p12^I$ localizes to the endoplasmic reticulum and Golgi, where it associates with two resident calcium-binding proteins, calreticulin and calnexin (Ding et al., 2001) causing sustained release of calcium (Ding et al., 2002), leading to activation of nuclear factor of activated T cell (NFAT)-mediated transcription (Albrecht et al., 2002). $p12^I$ also binds the calcium/calmodulin-dependent serine/threonine phosphatase, calcineurin (Kim et al., 2003). We have recently reported that $p12^I$ expression in Jurkat T cells alters the expression of several calcium-regulated genes (Nair et al., 2005). A surprising finding in this report was the observation that p300 mRNA was increased in Jurkat and primary human T cells during $p12^I$ expression (Nair et al., 2005). Based on this, we hypothesized that the transcription of p300 is calcium dependent and that sustained low magnitude increase in intracellular calcium concentration would enhance the transcription of p300.

Herein, we report that HTLV-1 $p12^I$ increases the expression of p300 mRNA and protein in T cells. The enhanced p300 expression was calcium dependent, but calcineurin independent. We demonstrate that ionomycin, a well-characterized calcium ionophore that triggers calcium release in Jurkat T cells, causes increased mRNA and protein levels of p300 sufficient to enhanced transcription from p300-dependent reporter genes. Furthermore, this p300-dependent transcriptional activity could be blocked by BAPTA-AM, a known calcium chelator, whereas cyclosporine A, a calcineurin inhibitor, did not have any effect on the p300-dependent transcriptional activity. In addition, using an ER-localization-deficient mutant of HTLV-1 $p12^I$, we demonstrate that ER localization of $p12^I$ is required for its ability to increase p300. In a dose-dependent manner, the expression of $p12^I$ reversed HTLV-1 $p30^{II}$-mediated repression of HTLV-1 LTR-driven transcription. As $p30^{II}$ is a p300-binding protein, these data illustrate that HTLV-1 accessory proteins may act together to balance viral expression. Collectively, HTLV-1 $p12^I$ appears to regulate the expression of the key cellular transcriptional co-activator p300 through the ubiquitous second messenger calcium to modulate viral gene expression and promote long-term T lymphocyte survival.

Results

HTLV-1 $p12^I$ enhances expression of p300

We have reported that the expression of $p12^I$ in Jurkat T cells and primary CD4 T lymphocytes is associated with enhanced expression of p300 mRNA and increased p300-mediated transcription in Jurkat T cells (Nair et al., 2005). To test whether expressing $p12^I$ in T cells results in increased p300 itself, we performed western immunoblot assays from Jurkat T cells spin infected with recombinant lentiviral vectors. Our data indicated that Jurkat T cells expressing $p12^I$ had ~2.1-fold higher protein levels of p300 compared to mock vector-infected control Jurkat T cells (Figs. 1A and B).

Sustained low magnitude increase in intracellular calcium enhances the levels of p300 by increasing its transcription

Previous reports from our laboratory demonstrated that HTLV-1 $p12^I$ increases cytosolic calcium by enhancing release from ER stores (Ding et al., 2002). Increased intracellular calcium levels have been extensively studied using calcium ionophores, such as ionomycin (Liu and Hermann, 1978). Ionomycin is routinely used for investigating calcium-mediated T cell activation and proliferation. At high concentrations (0.5–2.0 μM), typically used to study proliferative responses of cells to calcium, ionomycin mediates passive calcium influx by directly inserting into the plasma membrane (Donnadieu et al., 1995). However, at 10- to 100-fold lower concentrations (50–

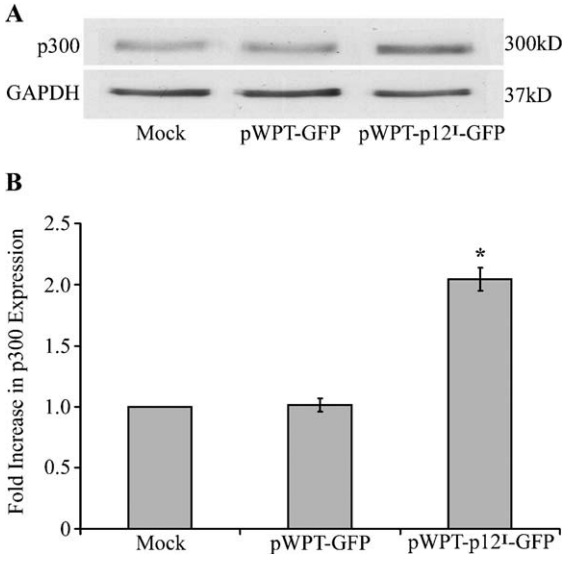

Fig. 1. HTLV-1 $p12^{I}$ enhances p300 protein levels in Jurkat T lymphocytes. (A) Jurkat T lymphocytes (2×10^6) were spin infected with lentiviral vectors expressing $p12^{I}$ at an MOI of 5. Total protein was extracted and western immunoblot analysis for the detection of p300 was performed. Jurkat T cells expressing $p12^{I}$ demonstrated increased protein levels of p300. (B) Densitometric analysis of radiograph was performed using Gel pro analyzer software and normalized to GapDH. Jurkat T cells expressing $p12^{I}$ showed ~2.1-fold increased expression of p300. Statistical analysis was performed using Student's *t* test, $P < 0.05$.

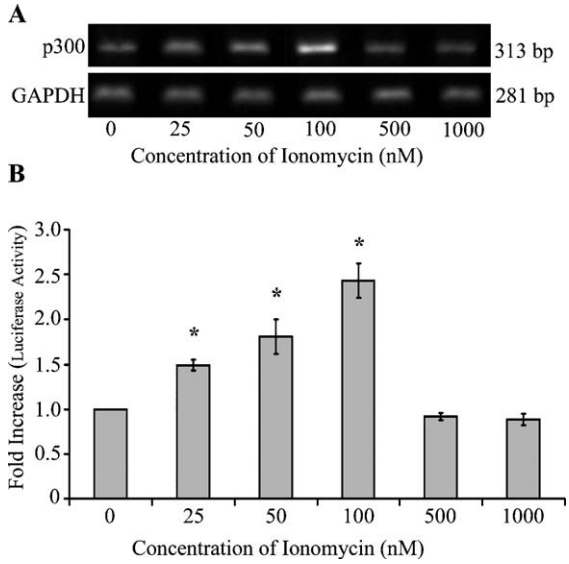

Fig. 2. Sustained low magnitude increase in intracellular calcium concentration enhances transcription of p300. (A) Jurkat T cells (1×10^6 per ml) were stimulated with varying concentrations of ionomycin for 36 h. Total cellular RNA was extracted and semi-quantitative RT-PCR was performed to identify the mRNA levels of p300. There was a dose-dependent increase in p300 expression in Jurkat T cells stimulated with low concentrations of ionomycin (25, 50, and 100 nM). (B) Densitometric analysis was performed using alpha imager software and normalized to GAPDH. Dose-dependent increases (up to 2.4-fold) in p300 mRNA were observed in Jurkat T cells stimulated with 100 nM ionomycin. Statistical analysis was performed using Student's *t* test, $P < 0.05$.

100 nM), ionomycin binds cellular endomembranes and increases cytosolic calcium by depletion of intracellular calcium stores and capacitative calcium entry (Donnadieu et al., 1995; Putney, 1990). Thus, stimulation of T cells with 50–100 nM of ionomycin causes calcium influx similar to HTLV-1 $p12^{I}$ (Ding et al., 2001).

To test if low magnitude increase in intracellular calcium mediated through ionomycin enhances the expression of p300, we performed semi-quantitative RT-PCR on total RNA extracted from Jurkat T cells stimulated with ionomycin. The cells were stimulated with 25, 50, 100, 500 nM, and 1 μM of ionomycin for 36 h before RNA extraction. Our data indicated that p300 expression was increased in a dose-dependent manner up to 100 nM of ionomycin in Jurkat T cells, 1.5, 1.8, and 2.4, respectively (Figs. 2A and B). p300 mRNA were not significantly altered at higher concentrations of ionomycin (500 nM and 1 μM) due to reduced cell viability at these higher concentrations, a known property of high dose ionomycin treatments.

To investigate if increased levels of p300 mRNA correlated with a corresponding increase in protein levels of p300, we performed western immunoblot assays using Jurkat T cells stimulated with these same concentrations of ionomycin. Our data indicated a dose-dependent increase in p300 in Jurkat T cells stimulated with these lower concentrations of ionomycin. The fold increase in p300 protein levels in cells treated with 25, 50, and 100 nM of ionomycin were 1.2, 1.6, and 2.4, respectively (Figs. 3A and B). Densitometry values were normalized to constitutively expressed GAPDH in both RT-PCR and western immunoblot assays.

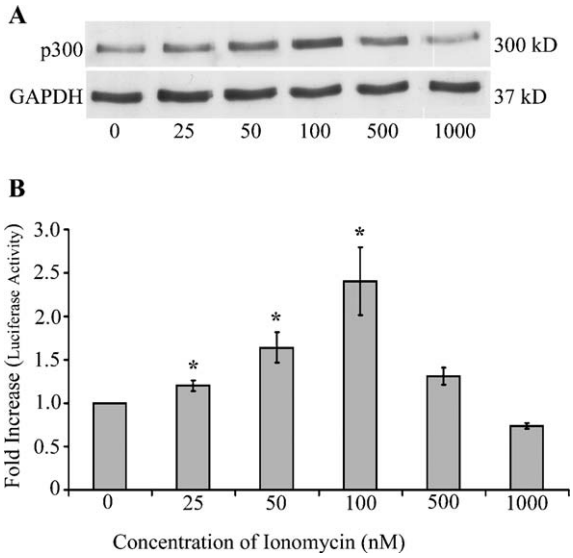

Fig. 3. Enhanced mRNA levels of p300 correlates with increased protein levels of p300. (A) Jurkat T cells (1×10^6 per ml) were stimulated with varying concentrations of ionomycin for 60 h. Total protein was extracted and western immunoblot analysis for the detection of p300 protein. There was a dose-dependent increase in p300 expression in Jurkat T cells stimulated with low concentrations of ionomycin (25, 50, and 100 nM). (B) Densitometric analysis of radiograph was performed using Gel pro analyzer software and normalized to GAPDH. Dose-dependent increases (up to 2.5-fold) of p300 were observed in Jurkat T cells stimulated with 100 nM ionomycin. The protein levels were in parallel with mRNA levels observed at similar concentrations of ionomycin. Statistical analysis was performed using Student's *t* test, $P < 0.05$.

Low dose ionomycin enhances p300-mediated transcription

To investigate whether the increased levels of p300 observed during low dose ionomycin treatment resulted in enhanced transcription from p300-dependent promoters, we tested the effect of low dose ionomycin in a VP16-mediated transactivation assay (Nair et al., 2005). Our data indicated that low dose ionomycin elicited a dose-dependent increase in VP16-mediated transactivation (Fig. 4A). Approximately 3.3- to 4.4-fold higher luciferase values were observed at 50 and 100 nM concentrations of ionomycin (Fig. 4A). To further confirm that this increased luciferase activity was specific to increased levels

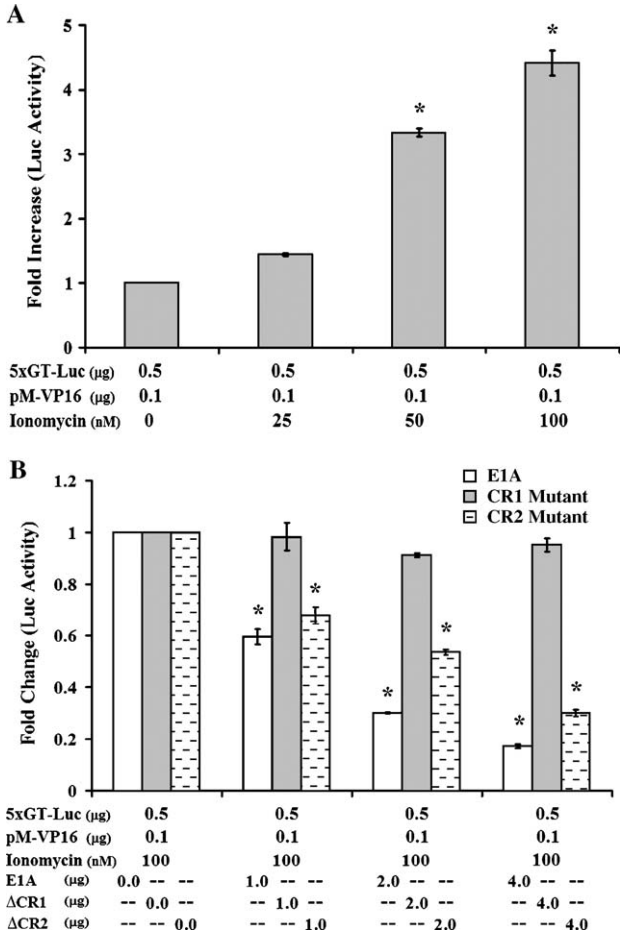

Fig. 4. Ionomycin-mediated increase in p300 influences transcription. (A) 2×10^6 Jurkat T cells were transfected with 5xGT-Luc and pM-VP16. The transfected cells were maintained in cRPMI medium containing increasing concentrations of ionomycin. The luciferase activity was tested 60 h post-transfection. A dose-dependent increase in VP16-mediated p300-dependent luciferase activity was observed with increasing concentrations of ionomycin. (B) The increase in VP16-mediated luciferase activity was confirmed to be p300 dependent by transfecting wild-type and mutants of adenoviral E1A protein. Two million Jurkat T cells were transfected with 5xGT-Luc, pM-VP16, and increasing concentrations of wild-type or mutants of adenoviral E1A protein. The transfected cells were incubated in cRPMI medium supplemented with 100 nM ionomycin. ΔCR1 mutant of E1A is incapable of binding p300 whereas ΔCR2 mutant is capable of binding p300 but not retinoblastoma protein. VP16-mediated p300-dependent luciferase activity was inhibited in a dose-dependent fashion in the presence of wild-type as well as ΔCR2 mutant of E1A. No effect on luciferase activity occurred in the presence of ΔCR1 mutant of E1A.

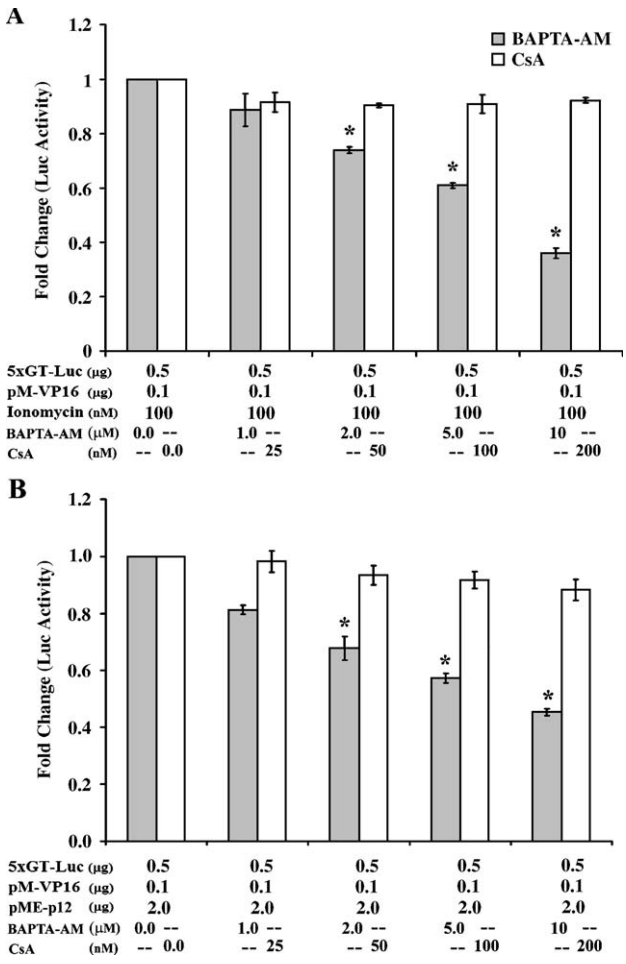

Fig. 5. Enhanced expression of p300 is calcium dependent, but calcineurin independent. (A) Jurkat T cells (2×10^6) were transfected with 5xGT-Luc and pM-VP16 and incubated in cRPMI medium supplemented with 100 nM ionomycin. The cells were treated with increasing concentrations of BAPTA-AM or CsA. A dose-dependent reduction in VP16-mediated p300-dependent luciferase activity was noticed in the presence of BAPTA-AM whereas no significant difference in luciferase activity was observed in the presence of CsA. (B) Jurkat T cells (2×10^6) were transfected with 5xGT-Luc, pM-VP16, and pME-p12[I] and incubated in cRPMI medium. The cells were treated with increasing concentrations of BAPTA-AM or CsA. A dose-dependent reduction in VP16-mediated p300-dependent luciferase activity was evident in the presence of BAPTA-AM, whereas no significant difference in luciferase activity was observed in the presence of CsA.

of p300, we co-transfected and adenoviral E1A expressing plasmid, which completely inhibits p300 transcription by directly binding to the transcriptional co-adaptor. Plasmids expressing wild-type E1A or mutants of E1A, including ΔCR1 that contains a mutation in the p300-binding region and ΔCR2 containing a mutation in the retinoblastoma-binding region were transfected with the luciferase reporter plasmid and pM-VP16. Wild-type E1A and the ΔCR2 mutant of E1A inhibited the transactivation of VP16 in a dose-dependent manner (Fig. 4B). Our data indicated up to 82.3% reduction in luciferase activity with wild-type E1A and approximately 70.0% reduction with the ΔCR2 mutant of E1A (Fig. 4B). The ΔCR1 mutant, which is unable to bind p300, did not have any effect on luciferase activity, indicating the p300-dependent nature of

VP16-mediated transactivation (Fig. 4B). These findings indicate that low magnitude increases in intracellular calcium enhance p300 expression to functionally significant levels.

Enhanced expression of p300 is calcium dependent, but calcineurin independent

We then confirmed that $p12^I$ enhancement of p300-dependent VP16-driven transactivation was mediated by calcium by transfecting our $p12^I$ expression plasmid into Jurkat T cells in the presence of the calcium chelator BAPTA-AM (Fig. 5). In addition, we also stimulated Jurkat T cells with ionomycin with increasing concentrations of BAPTA-AM. p300-dependent VP16-mediated transactivation was inhibited in the presence of BAPTA-AM in a dose-dependent manner in both $p12^I$-transfected and ionomycin-stimulated Jurkat T cells. We observed a 55% reduction in luciferase activity with 10 μM BAPTA-AM in Jurkat T cells expressing $p12^I$ and 64% reduction in luciferase activity in Jurkat T cells stimulated with 100 nM ionomycin, respectively (Figs. 5A and B). We then blocked the calcium-dependent phosphatase calcineurin by using cyclosporin A. Calcineurin is the key enzyme required for NFAT translocation to the nucleus. Both $p12^I$-expressing and ionomycin-stimulated Jurkat T cells exhibited no significant difference in luciferase activity with increasing concentrations cyclosporin A up to 200 nm (Figs. 5A and B). BAPTA-AM alone did not have any significant effect on luciferase expression without the presence of calcium. These data were consistent with our previous data and indicated that the expression of p300 is dependent on calcium, but not calcineurin.

The mechanisms of transcriptional regulation of p300, including transcription factors that cooperatively bind the p300 promoter, have not been defined. To gain initial insight into predicted transcription factor binding sites in the p300 promoter, we located the mRNA nucleotide sequence for p300 (NM_001429) using a Web-based resource (NCBI nucleotide database). Then proceeding from transcriptional start site of the p300 mRNA to the beginning of the mRNA message, we analyzed 403 nucleotide sequences to predict transcription factor binding sites in the p300 promoter. Nucleotide sequences for consensus or putative binding sites for a number of transcription factor family members are represented in the promoter region of p300. These include transcription factors that are known to respond to calcium signaling such as NF-κB and NFAT (Im and Rao, 2004; Quintana et al., 2005) (Table 1).

Localization of $p12^I$ to ER is required for enhanced expression of p300

Previous studies from our laboratory demonstrated that ER localization of HTLV-1 $p12^I$ is critical for the release of calcium its enhancement of NFAT-mediated transcription (Ding et al., 2003). We therefore tested whether ER localization of $p12^I$ is required for its ability to enhance the expression of p300. An N- and C-terminal deletion mutant of $p12^I$ containing amino acids 15–47 localizes to the nucleus and the ER localization of this mutant requires the addition of an ER targeting signal KKLL (Ding et al., 2003). This mutant allowed us the opportunity to test if ER localization of $p12^I$ is required to enhance p300-dependent transactivation by transfecting Jurkat T cells with equal amounts of either the empty vector, wild-type $p12^I$, 15–47 mutant of $p12^I$, or the ER redirected 15-47-KKLL chimeric mutant along with the luciferase reporter and Gal4-VP16 expression plasmids. A marked reduction in the luciferase activity, up to 70%, was observed in Jurkat T cells expressing the 15–47 mutant of $p12^I$, suggesting that ER localization of

Table 1
Selected putative transcription factor sites in p300 promoter[a]

Transcription factor	Nucleotide position	Matrix simulation score	Sequence
Ets—family member ELF-2 (NERF1a)	10–26	0.910	cgaggaGGAAgaggttg
Human zinc finger protein ZNF35	12–24	0.963	aggaggAAGAggt
b zip family, induced by ER damage binds in association with NF-Y	67–81	0.945	ccgCCACggccggcc
STAT6: signal transducer and activator of transcription 6	107–125	0.963	gcgaaTTCCcgagaactcg
Ikaros 1, potential regulator of lymphocyte differentiation	112–124	0.934	tctcGGGAattcg
HMGI (Y) high-mobility group protein I (Y) factor	113–125	0.953	gcgAATTcccgag
PAX6 paired domain and homeodomain	172–190	0.900	ggcttgggcCCAGgcccgg
Myc-associated zinc finger protein (MAZ)	188–200	0.910	gtgcGAGGggccg
Gut-enriched Krueppel-like factor	203–217	0.924	agaaaaggtaAGGGc
c-Rel	264–278	0.914	aaaggaacTTCCccc
NF-kappaB	264–278	0.964	ggGGGAagttccttt
Zinc finger transcription factor ZBP-89	270–292	0.971	acttcccccaCCCCctcgggtgc
Erythroid Krueppel like factor (EKLF)	277–289	0.915	cccgaggGGGTgg
Myeloid zinc finger protein MZF1	327–333	0.985	gcGGGGa
Nuclear factor of activated T cells	368–378	0.975	cgagGAAAacc
Brn-2, POU-III protein class	391–403	0.946	cggccattttTAATtcttt

[a] mRNA nucleotide sequence for p300 (NM_001429) obtained from NCBI Web-based resource (http://www.ncbi.nlm.nih.gov/). Preceding from the predicted start site of p300 mRNA, 403 nucleotide sequences were analyzed using commercially available software (Genomatix: http://www.genomatix.de/) to analyze and predict transcription factor binding sites in the promoter region in the p300 gene (chromosome 22, contig-NT_01150.09, *Homo sapiens* chromosome 22). Base pairs in capital letters denote the core sequence used by MatInspector (Genomatix). Matrix simulation scores with great than 0.90 were selected from 66 total predicted transcription factor binding sites, based from a 1.00 core simulation score of known nucleotide sequence binding sites.

p12^{I} is critical for its ability to enhance the expression of p300 (Fig. 6). This tenet was further supported by the ability of the ER-targeted 15-47-KKLL mutant of p12^{I} to partially (80% of the wild-type levels) restore the luciferase activity (Fig. 6).

HTLV-1 p12^{I} inhibits the transcriptional repression of p30^{II} on HTLV-1 LTR

p300 plays a crucial role in the regulation of viral gene transcription from HTLV-1 LTR in infected cells by forming complexes with other transcriptional factors (Lemasson et al., 2002). HTLV-1 Tax transactivates LTR-driven transcription of HTLV-1 genes, in part, through its interaction with the p300 (Bex and Gaynor, 1998). In addition, studies from our laboratory have demonstrated that an accessory protein of HTLV-1, p30^{II}, inhibits transcription of viral genes from the HTLV-1 LTR (Zhang et al., 2001). This inhibition involves the interaction of p30^{II} with p300 and making it unavailable for transcriptional co-activation at the viral LTR (Zhang et al., 2001). By transiently transfecting increasing concentrations of pCMV-p300 with constant amounts of pME-p30^{II}HA plasmid, studies from our laboratory demonstrated that p300 expression reverses the p30^{II}-dependent repression of LTR-luciferase reporter gene activity (Michael et al., submitted for publication).

Because p12^{I} has the ability to increase the levels of p300, we hypothesized that p12^{I} expression would reverse the inhibitory effect of p30^{II} on HTLV-1 LTR-driven transcription. To test our hypothesis, we transfected increasing amounts of our pME-p12^{I}HA plasmid concurrently with a constant amount of pME-p30^{II}HA plasmid in the presence of an HTLV-1 LTR-responsive reporter gene. Our data indicated that p12^{I} expression reverses the p30^{II}-dependent repression of LTR-luciferase reporter gene activity in a dose-dependent manner (Fig. 7). We did not observe alterations in the cellular localization of p30^{II} in the presence of p12^{I} (data not shown). To test if the p12^{I} block of p30^{II} repression of LTR reporter gene activity was p300 dependent, we co-transfected an adenoviral E1A expression plasmid along with pME-p12^{I}HA and pME-p30^{II}HA plasmids and demonstrated that the expression of E1A blocked the effect of p12^{I} on p30^{II}-mediated LTR repression (Fig. 7). These data further confirmed that HTLV-1 p12^{I} inhibits the transcriptional repression of p30^{II} on the HTLV-1 LTR in a p300-dependent manner and establishes that HTLV-1 accessory proteins have the capacity to act cooperatively to modulate viral gene expression.

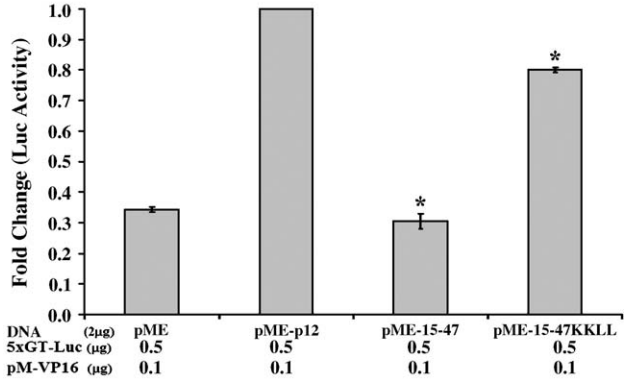

Fig. 6. Accumulation of p12^{I} to ER is required for enhanced expression of p300. Jurkat T cells (2×10^{6}) were transfected with 5xGT-Luc, pM-VP16, along with either of the following plasmids—pME control vector, pME-p12^{I}, pME-15-47, or pME-15-47KKLL, and then incubated in cRPMI medium. pME-15-47, which accumulates in the nucleus of transfected cells (Ding et al., 2002), exhibits a marked reduction in the VP16-mediated p300-dependent luciferase activity. The activity was partially restored by redirecting the protein to the ER.

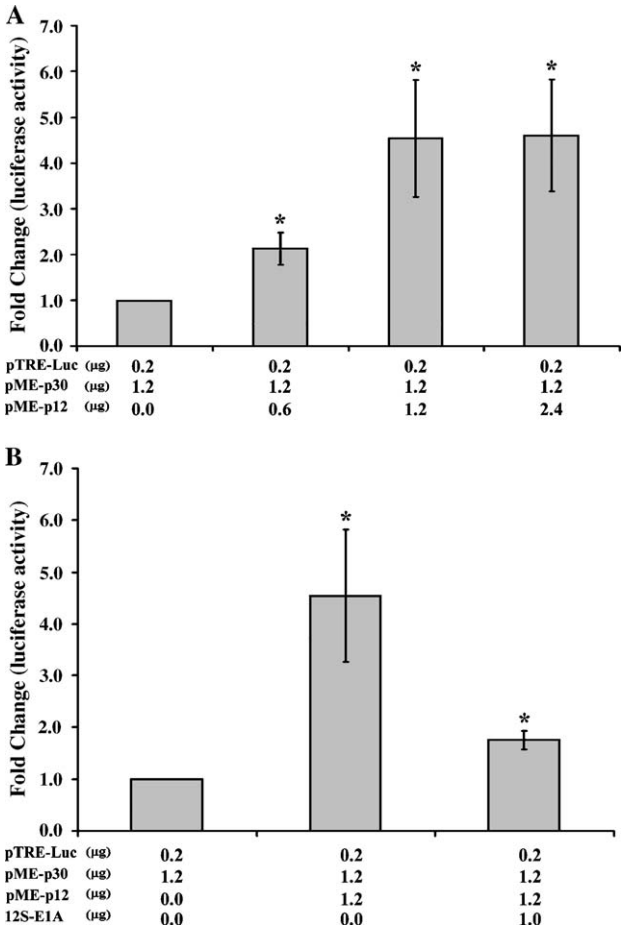

Fig. 7. HTLV-1 p12^{I} partially inhibits the transcriptional repression of p30^{II} on HTLV-1 LTR. (A) Jurkat T cells (2×10^{6}) were transfected with pTRE-Luc, pME-p30^{II} and increasing concentrations of pME-p12^{I} and then incubated in cRPMI medium. A dose-dependent increase in luciferase activity was observed with increasing concentrations of p12^{I}. (B) To confirm that the increased luciferase activity from pTRE-Luc was dependent on increased levels of p300, we transfected adenoviral E1A protein with the above-described plasmids. These data indicated a marked reduction in luciferase activity in the presence of E1A.

Discussion

HTLV-1, a human deltaretrovirus associated with lympho-proliferative disorders, encodes nonstructural or "accessory" proteins that modulate viral and cellular gene expression by interaction with p300 or through calcium-dependent mechanisms (Michael et al., 2004; Albrecht and Lairmore, 2002). One such protein encoded in pX ORF I of HTLV-1 is p12^{I}, which

localizes to the endoplasmic reticulum and Golgi, where it associates with two resident calcium-binding proteins, calreticulin and calnexin (Ding et al., 2001), causing sustained release of calcium (Ding et al., 2002) leading to activation of nuclear factor of activated T cell (NFAT)-mediated transcription (Albrecht et al., 2002). We have reported recently that $p12^{I}$ expression in Jurkat T cells alters the expression of several calcium-regulated genes (Nair et al., 2005). Unexpectedly, we observed that p300 mRNA was increased in T cells during $p12^{I}$ expression (Nair et al., 2005). We therefore hypothesized that the transcription of p300 is calcium dependent and that sustained low magnitude increase in intracellular calcium concentration would enhance the transcription of p300. Our data presented herein is the first to demonstrate that p300, an important co-activator of transcription, is responsive to sustained low magnitude increases in intracellular calcium. Because p300 is limited in cells, the expression of p300 in long-lived lymphocytes, such as memory T cells, would be particularly important for their survival. Interestingly, HTLV-1 has been demonstrated to target memory T cells (Yoshie et al., 2002; Lal et al., 1992). We demonstrate that ER localization of $p12^{I}$ is required for its ability to increase p300. In addition, our data indicate that $p12^{I}$ reverses the repression of HTLV-1 LTR-driven transcription by HTLV-1 $p30^{II}$. HTLV-1 $p12^{I}$-mediated enhancement of p300 expression represents a novel mechanism of regulation of cellular gene expression by viral proteins. By increasing calcium-mediated signaling HTLV-1 $p12^{I}$ enhances the expression of p300, which would modulate viral gene expression and favor cell survival. Collectively, our data provide new insight into HTLV-1-mediated gene expression and calcium signaling as a mechanism of transcriptional regulation of p300.

Calcium is a universal and highly versatile intracellular messenger, which plays a critical role in many varied biological processes such as cell proliferation, cell cycle, transcription, signal transduction, and apoptosis (Lewis, 2001). Calcium fluxes in mammalian cells are achieved by altering the amplitude and spatial distribution of intracellular calcium (Berridge et al., 1999, 2000; Bootman et al., 2001). Calcium mobilization in lymphocytes is accomplished by binding of inositol 1,4,5-trisphosphate (IP_3) to its receptor in the ER membrane and subsequent rapid but transient release of Ca^{2+} from ER stores (Berridge, 1993). Alternatively, sustained extracellular Ca^{2+} influx across the plasma membrane, by store-operated or capacitative Ca^{2+} entry, is activated by depletion of intracellular Ca^{2+} stores and operated through store-operated Ca^{2+} channels (SOC) or calcium release-activated Ca^{2+} channels (CRAC) (Clapham, 1995; Parekh and Penner, 1997; Putney, 1990).

Based on DNA microarray analysis, Feske et al. (2001) demonstrated that Ca^{2+} signals modulate the expression of various genes involved in transcription, including c-Myc, c-Jun, CREM, NFAT4, FosB, E2F3, IRF-1, IRF-2, NF-IL3A, Fra-2, FLI1, and SMBP2. Calcium-dependent activation of a wide variety of transcription factors, such as NFAT, NFκB, Elk-1, Nur77, AP-1, ATF-2, and CREB, occurs through calmodulin-dependent protein kinases and phosphatases (Aramburu et al., 2000; Rao et al., 1997; Tokumitsu et al., 1995). Whereas a small transient spike of Ca^{2+} increase by store depletion activates signaling pathways and transcription factors such as NFκB and JNK (Dolmetsch et al., 1997), capacitative calcium entry and a sustained Ca^{2+} increase are necessary to activate other transcription factors, such as NFAT (Dolmetsch et al., 1997, 1998; Li et al., 1998).

To date, p300 has not been identified as a calcium-responsive gene. In addition, the transcriptional regulation of p300 and detailed examination of transcription complexes operative in regulating p300 have not been reported. We used the mRNA nucleotide sequence to predict transcription factor binding sites in the p300 promoter. Our analysis indicates that the p300 promoter contains binding sites for a number of transcription factor family members known to respond to calcium signaling such as NF-κB and NFAT (Im and Rao, 2004; Quintana et al., 2005), suggesting that calcium signaling has a role in replenishing limiting amounts of cellular p300.

Typically, the role of calcium in various signal transduction pathways has been investigated using relatively high concentrations (0.5–2.0 μM) of calcium ionophores, such as ionomycin, for short periods of time (6–18 h). This stimulation condition simulates calcium-dependent T cell activation upon antigen binding to the T cell receptor; however, such stimulation protocols do not simulate the effect of prolonged stimulation with calcium. Higher concentrations of ionomycin are cytotoxic, limiting the possibility of long-term stimulation. This might explain why previous studies did not identify p300 as a calcium-regulated gene. At low concentrations (50–100 nM), ionomycin binds cellular endomembranes and increases cytosolic calcium concentration by depletion of intracellular calcium stores and capacitative calcium entry (Donnadieu et al., 1995; Putney, 1990). Low concentrations of ionomycin therefore appear to simulate the effect of HTLV-1 $p12^{I}$, which causes sustained increases in intracellular calcium concentration (Ding et al., 2002).

A number of viruses encode proteins that modulate cellular Ca^{2+} homeostasis to regulate various aspects of viral pathogenesis (Ganem, 2001). Examples include hepatitis B virus X protein (HBx), which activates Ca^{2+} signaling through mitochondria to influence HBV replication (Bouchard et al., 2001), and strains of rotavirus that encode NSP4, a nonstructural glycoprotein that increases the cytosolic calcium in infected cells (Tian et al., 1995). In addition, Kaposi's sarcoma-associated herpesvirus (KSHV) mitochondrial protein K7 targets CAML, a cellular Ca^{2+}-modulating protein to increase cytosolic Ca^{2+} allowing the completion of lytic replication (Feng et al., 2002). Coxsackievirus protein 2B induces the influx of extracellular Ca^{2+} and releases Ca^{2+} from ER stores, modifying plasma membrane permeability to facilitate virus release (van Kuppeveld et al., 1997). HIV-1 Nef causes atypical IP_3R triggering of plasma membrane calcium influxes, independent of intracellular calcium stores (Manninen and Saksela, 2002). Furthermore, calcium plays a critical role in the replication cycles or pathogenesis of other viral infections including poliovirus, cytomegalovirus, vaccinia, and measles (Ruiz et al., 2000).

Our studies indicated that ablation of the acceptor splice site for the singly and doubly spliced ORF I mRNA in context to an infectious clone of HTLV-1 is associated with a reduction of viral infectivity in vivo (Collins et al., 1998) that can be model when resting T cells are used as targets in vitro (Albrecht et al., 2000). In addition, nucleotide sequence alleles of $p12^{I}$ including those that would result in specific residue changes associated with protein stability (lysine versus arginine at position aa 88) or truncation mutants have been reported in a minority (<7%) of HTLV-1-infected subjects with HAM/TSP, ATL, and asymptomatic patients (Martins et al., 2002; Furukawa et al., 2004; Iniguez et al., 2006). In this minority subset of infected persons, it appears that the presence of a lysine residue at position 88 of the protein is not a specific marker for HAM/TSP (Martins et al., 2002) and that truncations of $p12^{I}$ (e.g., premature stop codons leading to $p12^{I}$ variants from 82 to 86 amino acids in length) do not preclude transmission (Furukawa et al., 2004; Iniguez et al., 2006). These findings are consistent with our data using exogenously express truncation mutants of $p12^{I}$, which retain the ability to localized to subcellular compartments, and elicited calcium-mediated NFAT activation of T cells (Ding et al., 2002; Kim et al., 2003).

p300 forms complexes with other transcription factors at the HTLV-1 promoter and plays a critical role in the regulation of HTLV-1 transcription in infected T cells (Lemasson et al., 2002). HTLV-1 Tax activates the HTLV-1 LTR through its interaction with p300 (Bex and Gaynor, 1998; Kashanchi et al., 1998; Jiang et al., 1999) and directly interacts with p300 in an acetyltransferase/activator complex (Harrod et al., 2000). We have demonstrated that HTLV-1 $p30^{II}$ binds p300 at the highly conserved KIX region (Zhang et al., 2001). Intriguingly, the KIX domain is also the binding site of p300 for HTLV-1 Tax. At lower concentrations, $p30^{II}$ activates HTLV-1 LTR-mediated transcription and at higher concentrations, $p30^{II}$ represses LTR-mediated transcription (Zhang et al., 2000). In addition, $p30^{II}$ was able to disrupt CREB-Tax-p300 complexes bound to the viral 21-bp TRE repeats (Zhang et al., 2001). Thus, HTLV-1 $p30^{II}$ and Tax appear to compete with each other in modulating the transcriptional activity from the LTR, possibly through competitive binding to p300 (Michael et al., submitted for publication). Our data present herein indicate that $p12^{I}$ blocks the repression LTR-driven transcription by HTLV-1 $p30^{II}$. These findings indicate that $p12^{I}$-mediated enhancement of p300 expression may not only regulate cellular gene expression to promote lymphocyte survival, but may also cooperate with other viral proteins to regulate viral gene expression.

Overall, our study demonstrates that HTLV-1 $p12^{I}$ enhances the expression of p300, an important but limiting co-activator of transcription in T cells. Thus, HTLV-1 $p12^{I}$ alters calcium-dependent cell signaling to promote lymphocyte survival, a characteristic of HTLV-1-associated lymphoproliferative disorders. In addition, we demonstrate that $p12^{I}$ has the capacity to reverse the transcriptional repression of $p30^{II}$ on the HTLV-1 LTR in a p300-dependent manner, demonstrating that HTLV-1 accessory proteins have the capacity to act cooperatively to modulate viral gene expression.

Materials and methods

Cells and plasmids

Jurkat T cells (clone E6-1, catalog # TIB-152, American Type Culture Collection) were maintained in RPMI 1640 media (Invitrogen) supplemented with 15% FBS, 100 μg/ml streptomycin/penicillin, 2 mM L-glutamine, and 10 mM HEPES (Invitrogen). The pME-18S and pME-$p12^{I}$ plasmids (Mulloy et al., 1996) were provided by G. Franchini (National Cancer Institute, National Institutes of Health). The pME-$p12^{I}$ plasmid expresses the fusion protein of HTLV-1 $p12^{I}$ tagged with the influenza hemagglutinin (HA1) tag. Generation of $p12^{I}$ truncation mutants in the pME-18S vector was previously described (Ding et al., 2001). Mutant $p12^{I}$15-47KKLL, which was constructed by inserting an ER targeting (Gomord et al., 1999; Plemper et al., 2001) KKLL sequence, has been described previously (Ding et al., 2003). Plasmid p5XGT-TATA-Luc (P. Quinn, The Pennsylvania State University, Hershey, PA), contains five tandem Gal4 DNA-binding sequences upstream of a TATA box, derived from positions −264 to +11 of the phosphoenolpyruvate carboxykinase (PEPCK) gene in a luciferase reporter gene plasmid. The pRSV-β-Gal, 12SE1A, 12SE1A-ΔCR1, and 12SE1A-ΔCR2 (T. Kouzarides, University of Cambridge, Cambridge, UK) have been described previously (Zhang et al., 2000, 2001). The pTRE-Luc plasmid and pRSV-β-Gal have been described previously (Zhang et al., 2000, 2001). pME-$p30^{II}$HA plasmid, which was created by cloning the $p30^{II}$ sequence from HTLV-1 molecular clone, ACH with downstream influenza hemagglutinin (HA1) tag, into pME-18S plasmid (G. Franchini, National Cancer Institute) between 5′ *Eco*RI and 3′ *Not*I sites, has been described previously (Michael et al., submitted for publication). The lentiviral vector system for expression of HTLV-1$p12^{I}$ has been described (Nair et al., 2005).

Stimulation of Jurkat T cells

To identify the optimal concentration and time frame for calcium-mediated enhancement of the transcription of p300, Jurkat T cells were stimulated with 25, 50, 100, and 500 nM and 1 μM ionomycin. To identify mRNA levels of p300, RT-PCR was performed between 36 and 48 h post-stimulation, whereas western blot and luciferase assays were performed at 60 h post-stimulation. The calcium chelator BAPTA-AM [glycine, *N*,*N*-1,2-ethanediylbis(oxy-2,1-phenylene)-*bis*-*N*-2-(acetyloxy) methoxy-2-oxoethyl]-[*bis*(acetyloxy)methyl ester] (Molecular Probes) and calcineurin inhibitor cyclosporin A (Sigma) were added to Jurkat T cells 24 h post-transfection or post-stimulation with ionomycin for 1 h at 37 °C. Transfected cells were washed and resuspended in cRPMI, whereas cells stimulated with ionomycin were resuspended in cRPMI containing appropriate concentrations of ionomycin.

Semi-quantitative RT-PCR for p300

Total cellular RNA was isolated from Jurkat T lymphocytes stimulated with ionomycin, using RNAqueous as described by

the manufacturer (Ambion). One microgram RNA was reversed transcribed to cDNA as described by the manufacturer (Reverse Transcription system, Promega). cDNA from 100 ng of total RNA was then PCR amplified with AmpliTaq DNA polymerase (Perkin Elmer) using primers specific for p300 and GAPDH. The PCR primers are as follows: p300: GTAGCCTAAAAGACAATTTTCCTTG (forward), ATGTCAACCATCTGCACCAGTA (reverse); and GAPDH: TGCACCACCAACTGCTTAG (forward), GAGGCAGGGATGATGTTC (reverse). PCR was performed at multiple cycles to maintain the amplification in a linear range. PCR products were separated by agarose gel electrophoresis and densitometric analysis was performed using alpha imager spot densitometry (Alpha Innotech) as described previously (Albrecht et al., 1998). Densitometric values for p300 were normalized using the values for GAPDH. Statistical analysis was performed using Student's t test, $P < 0.05$. DNA contamination tested in samples by performing a control with no reverse transcriptase.

Analysis of p300 promoter

From the National Center for Biological Information (NCBI) Web-based resource (http://www.ncbi.nlm.nih.gov/), the mRNA nucleotide sequence for p300 (NM_001429) was obtained. Preceding from the predicted start site of p300 mRNA, 403 nucleotide sequences were analyzed using commercially available software (Genomatix: http://www.genomatix.de/) to analyze and predict transcription factor binding sites in the promoter region in the p300 gene (Chromosome 22, contig-NT_01150.09, *Homo sapiens* chromosome 22).

Western immunoblot assays

Western immunoblot assays for the detection of p300 were performed as described previously (Zhang et al., 2000, 2001). Briefly, cells were lysed in RIPA buffer containing phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS). Cell lysates were prepared by centrifugation at 14,000 rpm (Beckman) for 20 min at 4 °C. Equal amounts of proteins were mixed with Laemmli buffer (62.5 mM Tris [pH 6.8], 2% SDS, 10% glycerol, 0.2% bromophenol blue, 100 mM dithiothreitol). After boiling for 5 min, samples were electrophoresed through 5% polyacrylamide gels. The fractionated proteins were transferred to nitrocellulose membranes (Amersham Pharmacia Biotechnology) at 100 V for 1 h at 4 °C. Membranes were then blocked in Tris-buffered saline containing 5% nonfat milk and 0.1% Tween 20. p300 was detected with the rabbit anti-p300 primary antibody (Santa Cruz Biotechnology, catalog # SC-584), followed by an anti-rabbit immunoglobulin G (IgG)–horseradish peroxidase-conjugated goat antibody (Upstate). GAPDH, used as a normalization control, was detected using goat anti-GAPDH primary antibody (Santa Cruz) followed by an anti-goat immunoglobulin G (IgG)–horseradish peroxidase-conjugated donkey antibody (Upstate). Blots were developed using an enhanced chemiluminescence detection system (NEN Life Science). Densitometric analysis of radiograph was performed using Gel pro analyzer software (Media Cybernatic Inc.) and normalized to GAPDH. Protein detection was optimized to test proteins in the linear range of expression by testing at various protein concentrations and exposure times. Statistical analysis was performed using Student's t test, $P < 0.05$.

Reporter gene assays

Unless otherwise mentioned, all the transfections of Jurkat T cells were performed using Superfect transfection reagent according to manufacturer's instructions (Qiagen). Jurkat T lymphocytes (2×10^6) were transfected with 500 ng of 5xGT-luc, 100 ng of pM-VP 16,500 ng of pRSV-β-Gal in the presence or absence of increasing amounts of wild-type or mutant 12sE1A, and stimulated with appropriate amounts of ionomycin. When the effect of HTLV-1 $p12^I$ on p300 expression was tested, either pME-$p12^I$HA or pME-18s were transfected with the above described plasmids in the absence if ionomycin. To test the effect of $p12^I$ on HTLV-1 $p30^{II}$-mediated LTR repression, 0.2 μg of pTRE-Luc reporter plasmid was co-transfected with 1.2 μg pME-$p30^{II}$HA and increasing concentrations (0.0, 0.6, 1.2, and 2.4 μg) of pME-p12IHA using Lipofectamine Plus (Invitrogen). To test if the rescue of $p30^{II}$-mediated HTLV-1 LTR repression by p12I is p300 dependent, 1.0 μg E1A expression plasmid was also co-transfected. As an internal control for transfection efficiency, 0.1 μg of pRSV-β-Gal (Invitrogen) was used in each transfection. pME-18S was used as carrier DNA to equalize DNA concentrations for each transfection.

At 60 h post-transfection, cells were lysed with a commercial buffer (Promega) at room temperature for 15 min. Twenty microliters of each lysate was used to test luciferase reporter gene activity using an enhanced luciferase assay kit according to the manufacturer's protocol (Promega). Staining with 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal) (Sigma) and counting β-Gal-expressing cells were performed to normalize the transfection efficiency. The final concentration of ionomycin and BAPTA-AM used was chosen after multiple pilot experiments using different concentrations of these reagents. BAPTA-AM alone did not have any significant effect on luciferase expression without the presence of calcium. Data were expressed as mean of normalized luciferase activity in arbitrary light units (ALU) with standard error (SE) from a minimum of triplicate experiments. Statistical analysis was performed using Student's t test, $P < 0.05$.

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